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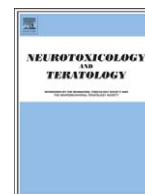
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Neurobehavioral teratogenicity of perfluorinated alkyls in an avian model[☆]

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ABSTRACT

Perfluorinated alkyls are widely-used agents that accumulate in ecosystems and organisms because of their slow rate of degradation. There is increasing concern that these agents may be developmental neurotoxins and the present study was designed to develop an avian model for the neurobehavioral teratogenicity of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS). Fertilized chicken eggs were injected with 5 or 10 mg/kg of either compound on incubation day 0. On the day of hatching, imprinting behavior was impaired by both compounds. We then explored underlying mechanisms involving the targeting of protein kinase C (PKC) isoforms (α , β , γ) in the intermedial part of the hyperstriatum ventrale, the region most closely associated with imprinting. With PFOA exposure, cytosolic PKC concentrations were significantly elevated for all three isoforms; despite the overall increase in PKC expression, membrane-associated PKC was unaffected, indicating a defect in PKC translocation. In contrast, PFOS exposure evoked a significant decrease in cytosolic PKC, primarily for the β and γ isoforms, but again without a corresponding change in membrane-associated enzyme; this likely partial, compensatory increases in translocation to offset the net PKC deficiency. Our studies indicate that perfluorinated alkyls are indeed developmental neurotoxins that affect posthatch cognitive performance but that the underlying synaptic mechanisms may differ substantially among the various members of this class of compounds, setting the stage for disparate outcomes later in life.

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1. Introduction

Perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) are prominent members of the family of perfluorinated alkyls (PFAs). These compounds are widely used for manufacturing non-stick coatings and stain repellents in fabrics, as well as in food-packaging and lubricants. PFAs are very poorly biodegradable and are accumulating in humans and ecosystems worldwide [17], extending

even to animals in remote locations [10]. Thus, despite recent efforts to reduce or phase out PFA production [9], these agents will persist in the environment [20,26].

Parallel to PFA bioaccumulation, toxicological studies in adult rats given high doses have shown damage to several internal organs, including the liver, kidneys and heart [43], preceded by substantial pathological changes in gene expression patterns [12]; parallel findings have been reported in zebra fish [29] and chicks [42]. In developing rodents, prenatal exposure to perfluorooctane sulfonate (PFOS) compromises survival rates and delays general growth and development [35]. In chicks, prenatal exposure to PFOS or perfluorooctanoic acid (PFOA) elicits reductions in hatchability, interference with pigmentation and pathological changes in the liver [24,38]. In our earlier work, we pointed out that the biochemical characteristics that underlie plumage pigmentation in the chick are also critical for nervous system development [38], suggesting that the PFAs might be developmental neurotoxins, a prediction we confirmed with *in vitro* models of neuronal development [30].

Studies in rodents similarly point to behavioral deficits in adulthood after neonatal exposure to PFOA or PFOS [16], reflecting an adverse impact on functioning of critical neurochemical events required for synaptic development and function [15]. Nevertheless, rodent models have inherent methodological shortcomings from confounding maternal

Abbreviations: ANOVA, analysis of variance; IMHV, intermedial part of the hyperstriatum ventrale; PFAs, perfluorinated alkyls; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonate; PKC, protein kinase C.

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factors, notably maternal care and mother–offspring interaction [27,32], and the so-called “litter effect,” [33], where animals within a given litter are not independent of each other. Obviously, in the chick model, these limitations do not apply, since there is no maternal interaction and every individual represents an independent sample; further, because the chick is more mature at hatching than are newborn rats or mice, cognitive behavior, in the form of imprinting performance, can be evaluated immediately, prior to any potential impact of prenatal treatment on feeding or other indirect contributors to behavioral abnormalities. A newborn chick tends to follow the first object it sees after hatching [21] and can thus be imprinted upon an artificial object, which then becomes a suitable subject for studying the effect of prenatal treatments on imprinting behavior [5,8,31]. We have already demonstrated how this parallels hippocampus-related visuospatial cognitive performance in rodents [39], involving in the chick the left intermedial part of the hyperstriatum ventrale (IMHV) [5,13]; specifically, cholinergic innervation in the left IMHV controls the chicks' perception of the imprinting stimulus [22,36]. Given the postulated involvement of cholinergic deficits in the long-term neurobehavioral effects of neonatal PFOA and PFOS exposure in rodents [16], in the present study we evaluated whether these agents have an adverse effect on imprinting performance in the chick model.

In addition to behavioral assessments, we also evaluated the impact of PFOA and PFOS exposure on the concentration of protein kinase C (PKC) isoforms, which play an integral mechanistic role in transducing cholinergic input involved in learning and memory. Specifically, the translocation of PKC from the cytoplasm to the membrane is required for imprinting [3,4,25,37]. In our studies, we obtained the brain samples immediately after the imprinting test so as to examine the ability of the imprinting stimulus to evoke PKC translocation. Indeed, in earlier work with other neurotoxicants known to target cholinergic function, we confirmed the direct relationship between adverse effects on PKC translocation and imprinting performance [14]. Here, we identified different patterns of effects of PFOA and PFOS on PKC-related mechanisms in association with impaired imprinting behavior.

2. Methods

2.1. Teratogen treatments

Fertile heterogeneous stock eggs (60 ± 3 g) of the Cobb 1 chicken broiler strain (*Gallus gallus domesticus*) were obtained from a commercial source and placed in an incubator. To introduce substances, a hole was drilled in the chorioallantois end (pointed end) of the shell and PFOA and PFOS (Sigma, Israel) were then administered before commencing incubation, i.e. on incubation day 0 (the PFOA in its free acid form and the PFOS in its salt form, due to dilution difficulties). The hole was then covered with an adhesive silicone glue cap (Medical Type A; Dow Corning, Midland, MI). Control eggs received equivalent volumes (1 ml/kg of egg) of corresponding vehicles: saline for PFOA and TWEEN (polyoxyethylenesorbitan monolaurate, undiluted) for PFOS. Preliminary studies were conducted over a range of doses (PFOA, 5–40 mg/kg of egg) to choose the optimal treatments that would produce behavioral defects without decreases in body weight or increased incidence of leg deformities [38]. The main studies were then conducted at doses of 5–10 mg/kg of egg for PFOA and PFOS. The eggs were incubated (model 1202 incubator; G.Q.F. Manufacturing Co., Savannah, GA) at 37.5 °C and 50–60% humidity, and were candled on incubation days 5, 12 and 19. At hatching, morphological and functional scoring was conducted using a four point scale, developed in our lab: 1. the chick lays on the floor 2. the chick stands hunched over and almost doesn't move 3. the chick shows some locomotion, 4. the chick moves around, exploring and pecking.

Fourteen to twenty-four hours posthatch, the chicks were trained to follow an imprinting object and were tested for performance as

described below. Afterward, the left IMHV was removed and taken for Western blot analysis of the levels of PKC isoforms.

2.2. Induction and testing of imprinting

As modified from earlier descriptions [23], the imprinting apparatus contained three 20-cm diameter running wheels with the sides covered in black PVC, permitting the chicks to see only forward or backward. The imprinting objects were an illuminated red box or a blue cylinder (both $15 \times 10 \times 18$ cm high), located 50 cm from the front open side of the running wheel, lit from within by a 40 W bulb with holes covered with red or blue filters, and rotated at 30 rpm. The chicks were hatched in total darkness and handling was done in the dark, aided by a dim green light, which has a minimal effect on imprinting [19]. All chicks were tagged and then transferred to an individual dark, enclosed wooden chamber warmed to 30 °C, where they were physically and visually isolated from each other. Fourteen to twenty-four hours posthatch, the chicks underwent 45 min of “priming,” a 30 min exposure to light (60 W bulb), followed by 15 min of darkness, conducted while recorded maternal calls were played continuously. Immediately afterward, they were placed individually on the running wheel for 60 min of training with either blue or red imprinting objects, and the wheel rotations running toward the imprinting object were recorded. After training, the chicks were returned to the enclosed chambers for 60 min, after which testing took place without any additional maternal calls.

There were four testing sessions in counterbalanced, randomized order, each lasting for 5 min; in two of the tests, the chick was allowed to run toward the imprinting rotating object and, in the other two, toward the control rotating object. For chicks trained to follow a red object, the red-light box was used as the imprinting object and the blue light box served as the control object; for the chicks trained to follow a blue object, these were reversed. Imprinting was then calculated as a preference score [31]: Preference score = Rotations toward the training light / (rotations toward the training light + rotations toward the control light). The expected range of the preference score is 0–1, where 0.5 indicates no imprinting, 1 represents maximal imprinting, and 0 represents avoidance (running away from the imprinted object).

Because this strain of chicken carries a sex-linked *early* feathering gene which allows for sexing of the chicks at hatching [11], the chicks' sex was known immediately following examination; because no significant differences were found, results were pooled for statistical analysis.

We also assessed locomotor activity of the different experimental groups (the number of rotations of the wheel made by the chick during training), since locomotion can by itself influence imprinting. The locomotor activity during imprinting testing (both training and control lights) was expressed as the total number of rotations of the wheel made by the chick.

2.3. Quantitative assessment of PKC isoforms

After behavioral testing, the brain was removed, and the left IMHV (2.5–3.0 mg) was quickly dissected [13]. Concentrations of the PKC isoforms in the cytosolic and membrane fractions of the IMHV were assayed as described in earlier publications [28,34,41]. Accordingly, only brief descriptions will be provided here.

The IMHV samples were sliced uniformly, homogenized, and then sedimented at $100,000 \times g$ for 1 h at 4 °C, after which the supernatant solution containing the cytosolic fraction was frozen. The membrane pellet was resuspended and digested with 0.5% Triton X-100 (Sigma), then sedimented as described above, and the supernatant solution was frozen. Western blot analysis was carried out by gel electrophoresis of 10–15- μ g aliquots of cytosolic and membrane proteins with specific primary antibodies for each PKC subtype. Antibodies to pPKC α and cPKC β II were purchased from Santa Cruz Biochemicals (Santa Cruz, CA) and PKC γ antibody 36G9, which recognizes chick

PKC γ [37], was specially produced for this study (E.A. Van der Zee). IgG HRP-conjugated secondary antibody (Bio-Rad, Hercules, CA) was used as described earlier [28,41]. As a calibration standard, every gel contained aliquots of a pooled homogenate combined from all the samples. This approach was preferable to using a “housekeeping” protein such as α -tubulin or β -actin, since neuroteratogens are known to influence neuromorphological development and thus alter expression of these cytoskeleton proteins, unrelated to the neurobehavioral effects linked to specific cell signaling pathways [1].

2.4. Data analysis

Data are presented as means and standard errors, with differences between treatments established by multivariate ANOVA, followed by Fisher's Least Significant Difference test for post hoc comparisons between groups. Significance for all tests was assumed at $p < 0.05$. For convenience, some results are presented as the percentage change from control values or as the percentage stimulation over basal PKC activity; however, statistical tests were always performed on the original data. Given the two different vehicles used to dissolve PFOA and PFOS, there were two sets of corresponding controls; further, the studies involved separate clutches of eggs for each of the PFAs, and accordingly, each treatment was compared only to its matched control group.

3. Results

On incubation day 19, fewer of the PFA-exposed eggs contained developing embryos as compared to the control groups, with reductions ranging from 30% to 50% (Fig. 1a). In addition, there were even further reductions in hatchability. However, among the chicks that hatched, there was no difference in weight (Fig. 1b) nor in the scoring for general morphology and function (Fig. 1c).

Control chicks showed typically-high imprinting scores [14] of about 0.8 (Fig. 2a). Chicks exposed to either of the PFAs lost about half their imprinting ability, although they still showed significant imprinting above the baseline score of 0.5. In general, PFOA had a greater effect than did PFOS. The effects did not show any sex preference (no interaction of treatment \times sex, not shown), so the effects of males and females are shown combined. The effects on imprinting were not secondary to loss of motor function, since overall activity levels in the imprinting apparatus were not significantly affected (Fig. 2b).

The concentrations of the PKC isoforms in subcellular fractions of the left IMHV are presented as the percent change from the corresponding control levels (Fig. 3). Exposure to PFOA produced an overall increase in cytosolic PKC involving all three isoforms but there was no corresponding change in membrane-associated PKC (Fig. 3a). In contrast, PFOS elicited significant overall reductions in cytosolic PKC with a distinct isoform preference of $\gamma > \beta > \alpha$; there were no significant effects on membrane-associated PKC.

4. Discussion

In the present study, embryonic exposure to PFOA and PFOS induced posthatch deficits in imprinting behavior in association with alterations in the concentrations of PKC isoforms within the left IMHV, the brain region in which imprinting is consolidated. Whereas the behavioral deficits were similar for both PFAs the changes at the molecular level differed, although as discussed below, the PKC changes for both agents are consistent with impaired expression and function of these key intermediates. Because the chick model, unlike mammalian models, does not involve maternal effects, our studies indicate that PFAs evoke neurobehavioral teratogenesis through direct effects on the developing brain, rather than through indirect compromise of maternal function or maternal–neonatal

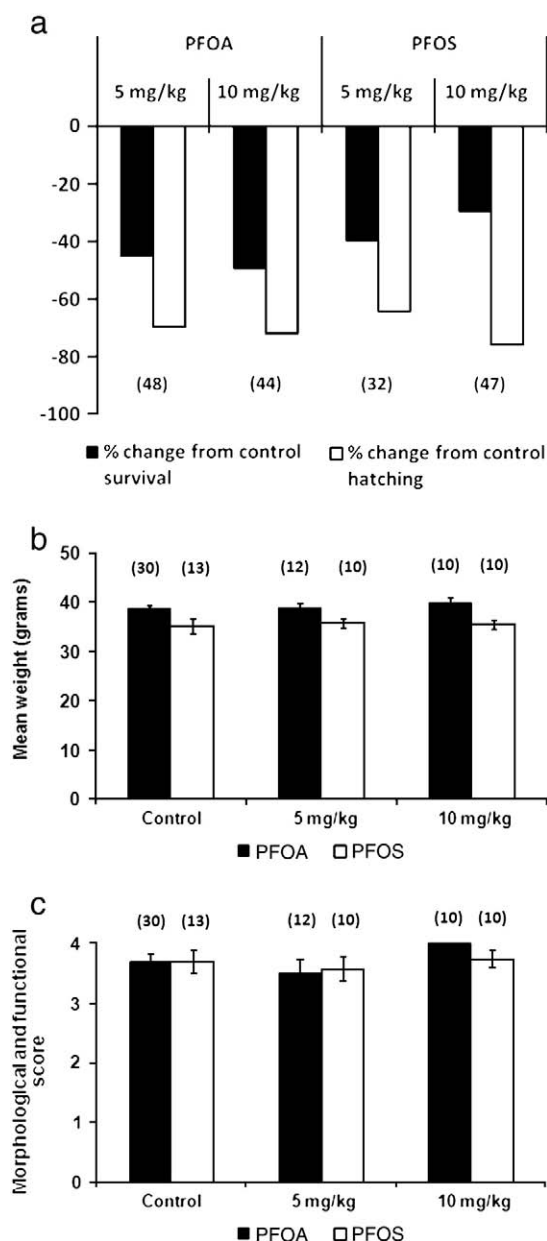


Fig. 1. a. Effect of pre-hatch exposure to PFOA and PFOS on (a) survival and hatching, (b) body weight at hatching and (c) morphological and functional score. Values in (b) and (c) represent mean \pm SE. Both PFOA and PFOS reduced survival and hatching ($p < 0.001$ by χ^2 test), whereas there were no significant differences in weight or morphological and functional score. Numbers of animals are shown in parentheses.

interactions; this reinforces conclusions drawn from in vitro models that similarly point to direct PFA actions on neuronal cell replication and differentiation [30].

In our previous studies with avian and rodent models, prenatal exposure to teratogens from different classes of compounds, all evoked deficits in the ability of PKC isoforms to translocate from the cytosol to the membrane, in association with their ability to impair cognitive performance: phenobarbital in the mouse [6]; heroin in the mouse [18] and chick [14]; and nicotine and the organophosphates, chlorpyrifos and sarin in the chick [14,40]. We were able to establish a mechanistic connection between the molecular and behavioral effects by administering targeted treatments that reversed the deficits [7,18,28]. Similarly, in the present study with PFOA and PFOS, we found alterations in PKC expression and translocation paralleling the behavioral deficits. However, the patterns seen with the PFAs were distinct from those of the other neuroteratogens. The previously-

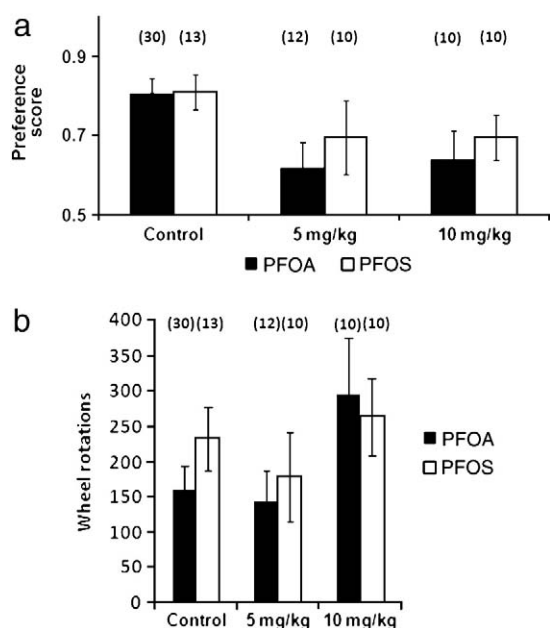


Fig. 2. Effect of pre-hatch exposure to PFOA and PFOS on (a) imprinting performance and (b) motor activity. Values represent mean \pm SE. In (a), controls show significant imprinting ($p < 0.001$ compared to the baseline value of 0.5); animals exposed to either PFOA or PFOS have significantly lower scores ($p < 0.05$) than corresponding controls but still have significant imprinting ($p < 0.05$ compared to the baseline value of 0.5). Numbers of animals are shown in parentheses. There were no significant differences in the total number of wheel rotations (b).

studied agents targeted primarily PKC γ , and to a lesser extent, PKC β , while largely sparing PKC α , and the deficits were much larger with respect to the membrane-associated fraction as distinct from cytosolic PKC [7,14,18,41]. Here, PFOA evoked large increases in the cytosolic fraction without a corresponding effect on membrane-associated PKC, and with little or no selectivity for any of the PKC subtypes. If the effectiveness of translocation was unaffected by PFOA, then the membrane-bound PKC should have increased by exactly the same proportion as for the cytosol; the fact that this did not occur again points to a defect in the ability to translocate PKC, similar to that seen with the other neuroteratogens that produce the same behavioral outcome. Nevertheless, the point remains that PFOA affects a wider spectrum of PKC subtypes than the other agents, and has unique effects on cytosolic PKC; PFOA thus may have a wider spectrum of adverse behavioral effects, a conclusion that clearly needs to be evaluated in future work.

Although both PFOA and PFOS evoked deficits in imprinting performance, the effects of PFOS on PKC isoforms differed from those of PFOA. Instead of causing a global increase in cytosolic PKC concentrations, PFOS elicited significant deficits; as with PFOA, these were unaccompanied by a corresponding change in the membrane-associated PKC fraction. Thus, PFOS decreased the cytosolic pool of PKC available for translocation but there was compensation so that the relative proportion of membrane translocation was higher, partially offsetting the drop in total PKC. If this difference from PFOA is mechanistically connected to the behavioral outcomes, then it would be expected that PFOS would produce somewhat smaller deficits, which was in fact the outcome that we observed. By extension, we would again predict fewer long-term behavioral deficits with PFOS than with PFOA. The idea that different PFAs might produce disparate neurodevelopmental outcomes is bolstered by recent *in vitro* findings demonstrating divergent effects on neuronal cell differentiation, particularly involving the emergence of the acetylcholine phenotype, the neurotransmitter most closely associated with imprinting [30]. In any case, the implication is clear that PFAs cannot simply be regarded as compounds that will all act

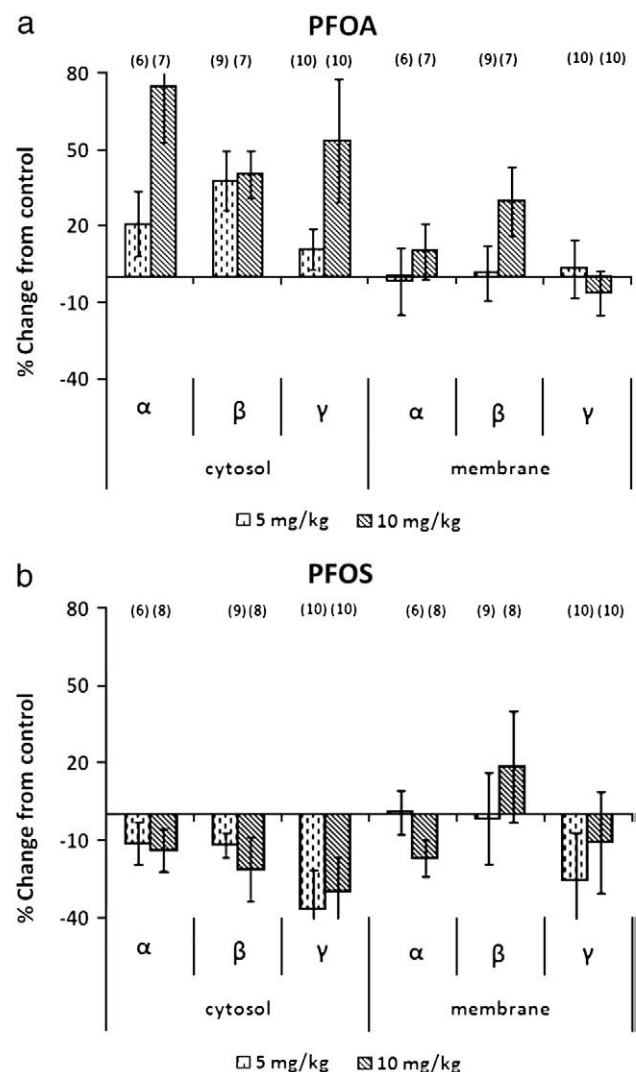


Fig. 3. Effect of pre-hatch exposure to (a) PFOA and (b) PFOS on PKC in subcellular fractions of the left IMHV. Scores represent mean \pm SE of the % change from control concentrations for each. PFOA produces a significant increase in cytosolic PKC (ANOVA main effect, $p < 0.0001$) without isoform specificity (no interaction of treatment \times isoform); there were no significant changes in the membrane fraction. PFOS produced a significant reduction in the cytosolic fraction (ANOVA main effect, $p < 0.0001$), with isoform selectivity of $\gamma > \beta > \alpha$ (treatment \times isoform, $p = 0.03$). Numbers of animals are shown in parentheses.

similarly on the developing nervous system but rather, they are likely to differ substantially in their net effects.

Although both PFOA and PFOS reduced hatchability, this factor does *not* translate well from avian to mammalian species. Avian embryos are extremely sensitive to even minor environmental perturbations, including vibration and movement, small changes in temperature and humidity, season, and clutch-to-clutch differences. In the present study, 20% of the control eggs were infertile or failed to hatch, a typical percentage for commercial hatcheries. In mammals, fetal resorption or intrauterine growth retardation occurs only at toxicant exposures that are considered hallmarks of high-dose toxicity but the same is not so for avian species, where effects on hatching often involve lower exposures than those required for neurobehavioral deficits. Thus, although our results provide interpretable cross-species results for neurodevelopmental endpoints, the nonspecific measures, such as hatching rate or malformations are of limited utility in comparing benchmarks for toxicant exposures. Another unique feature of the avian model is the fact that the egg is a closed system. Accordingly, we did not assess the posthatch levels of

PFOA and PFOS in the present study, but given that fact, combined with the resistance of these agents to chemical and biochemical breakdown, the body burden is likely to be the same as that given to the egg. Nevertheless, it would also be useful to assess the posthatch persistence of PFOA and PFOS and the relationship to more lasting neurobehavioral effects.

Production of PFOS in the U.S. was voluntarily discontinued by the manufacturer in 2002, whereas PFOA continued to be manufactured for several additional years [20,26]. Nevertheless, new members of the PFA class continue to emerge [2] and the legacy of the previously-used compounds will continue because of their long half-lives [20,26]. Our studies provide some of the first evidence that these compounds act directly as neuroteratogens and that the various members of the PFA class may differ in their ability to disrupt nervous system development.

Conflict of interest

No author has any conflict of interest to disclose.

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